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Immunoliposomes for the targeted delivery of antitumor drugs

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Abstract

This review presents an overview of the field of immunoliposome-mediated targeting of anticancer agents. First, problems that are encountered when immunoliposomes are used for systemic anticancer drug delivery and potential solutions are discussed. Second, an update is given of the in vivo results obtained with immunoliposomes in tumor models. Finally, new developments on the utilization of immunoliposomes for the treatment of cancer are highlighted.

Keywords: Anticancer agents; Immunoliposomes

Contents

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1. Introduction

An attractive strategy to enhance the therapeutic index of anticancer drugs is to specifically deliver these agents to tumor cells thereby keeping them away from non-malignant cells sensitive to the toxic effects of the drug. This would allow for more effective treatments achieved with doses that are better tolerated. Among the colloidal drug carrier systems proposed for site-specific drug delivery, liposomes have attracted considerable attention [1–6]. Liposomes consist of one or more concentric phospholipid bilayers, each enclosing an aqueous compartment. A large variety of therapeutically active molecules (e.g. antitumor drugs, oligonucleotides, DNA, enzymes, peptides and hormones) have been successfully incorporated in liposomes. Especially in the field of cancer chemotherapy, much effort has been invested to realize site-specific drug delivery with liposomal systems. Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells [6]. Several types of ligands have been used for this purpose, including antibodies or antibody fragments [7–10], vitamins [11], glycoproteins [12,13], peptides (RGD-sequences) [14,15], and oligonucleotide aptamers [16]. This review will mainly focus on the use of antibodies or antibody fragments to actively target liposomes, as this is the type of targeting ligand mostly used.

The first report on antibody-targeted liposomes came from Torchilin et al. exactly 2 decades ago [17]. These antibody-targeted liposomes (further referred to as immunoliposomes) were shown to be able to specifically bind to the antigen that is expressed on the target cells. Since then, several coupling techniques have been described for conjugating antibodies or their fragments to liposomes, each with their own advantages and drawbacks [9,18,19]. Many in vitro experiments have demonstrated highly specific binding of immunoliposomes to target cells. However, despite the excellent targeting properties in vitro, successful results on targeting of immunoliposomes in tumor models are, up to now, scarce.

In this review we aim to present the current status regarding the application of immunoliposomes for anticancer drug delivery in vivo. First, problems will be listed that are encountered when immunoliposomes are used for systemic anticancer drug delivery and potential solutions will be discussed. Second, an update will be given on the results obtained with immunoliposomes in animal models. Finally, special emphasis will be placed on highlighting new developments regarding the utilization of immunoliposomes for the treatment of cancer.

2. Immunoliposomes in vivo: many rivers to cross

Accessibility of tumor cells is a critical issue when immunoliposomes are to be targeted to tumors. Unlike some types of tumors (e.g. hematological

malignancies and tumors confined within a body cavity), many tumors are located at sites that are less accessible by intravenously (i.v.) injected liposomes. The process of targeted drug delivery with immunoliposomes can be roughly divided into two phases: the transport phase, in which the immunoliposomes travel from the site of administration (often i.v. administration) to the target cells, and the effector phase that includes the specific binding of immunoliposomes to the target cells and the subsequent delivery of entrapped drugs. This section will discuss the physiological and anatomical barriers to cross when immunoliposomes are administered i.v. and will point out potential solutions that have been investigated to enable immunoliposomes to fulfil their task.

2.1. Barriers encountered in the transport phase

2.1.1. Stability of immunoliposomes in the circulation

Directly after i.v. administration, immunoliposomes are exposed to a variety of factors that may compromise the integrity of the liposomal carrier. For example, adsorption of serum proteins to the liposome surface can induce premature leakage of entrapped compounds and liposome aggregation upon injection into the bloodstream [20–22]. Liposome aggregates will be rapidly cleared from the circulation by liver and spleen phagocytes and/or embolize lung capillaries. Furthermore, it has been demonstrated that naturally occurring antibodies (class IgM and IgG) with reactivity against the headgroup of phospholipids are present in serum of rabbits [23] and man [24]. Such naturally occurring polyclonal antibodies were able to activate complement via the alternative pathway, resulting in complement-mediated lysis of liposomes.

2.1.2. Clearance of immunoliposomes from the circulation

Perhaps the most important barrier limiting the usefulness of immunoliposomes for targeted drug delivery has been the rapid recognition and removal from the blood by cells of the mononuclear phagocyte system (MPS), particularly the macrophages in liver and spleen [25,26]. In addition, the presence of whole antibodies conjugated to the liposomal surface makes immunoliposomes highly susceptible to Fc-receptor-mediated phagocytosis and, as a result, even more prone to rapid clearance [25,27]. The Fc-receptor family, which is expressed by different cells of the MPS, binds the constant region (Fc) of antibodies resulting in internalization of antibody–opsonized complexes (e.g. bacteria) [28]. Similarly, immunoliposomes bearing whole antibodies are cleared rapidly due to exposed Fc parts [25,27,29].

The advent of so-called long-circulating liposomes produced by coating the liposome surface with the polymer poly(ethyleneglycol) (PEG), has revived interest in targeted drug delivery [30,31]. The PEG-coating has a dual effect. It sterically stabilizes the liposomal membrane against interactions with destabilizing and opsonic factors in vivo. As a consequence, PEG-coated liposomes show longer circulation times and reduced uptake by the MPS, relative to conventional liposomes. Although the exact mechanism behind the MPS avoidance phenomenon is still under discussion, it is thought that stabilization occurs by the formation of a highly hydrated shield of polymer molecules around the liposome which sterically inhibits both electrostatic and hydrophobic interactions of serum components with the liposomal bilayer [32,33]. Therefore, PEG–liposomes are often referred to as sterically stabilized liposomes.

There are different methods available for coupling antibodies to PEG–liposomes [58]. The pros and cons were recently discussed [9]. For attaching antibodies to the surface of PEG–liposomes, two main strategies have been followed: those in which the ligand is coupled directly to the liposome bilayer (Fig. 1A) and those in which the ligand is attached to the terminal end of PEG (Fig. 1B–D) [8,10,34]. The latter strategy yields protein present at the surface of the PEG coating. Indeed, it has been shown that the clearance rate of PEG–immunoliposomes is dependent on the antibody density at the liposome surface [8,10,35]. At low antibody density ($< \approx 50$ μg mAb/μmol PL), the PEG–immunoliposomes are cleared at rates only slightly more rapidly than those seen for antibody-free PEG–liposomes. At higher antibody density (> 100 μg mAb/μmol PL), the clearance becomes very rapid with half-lives of only a few minutes [8]. Likely, clearance is mediated by the exposed Fc-region of the whole antibodies conjugated to the PEG-terminal ends.

An interesting observation came from Maruyama

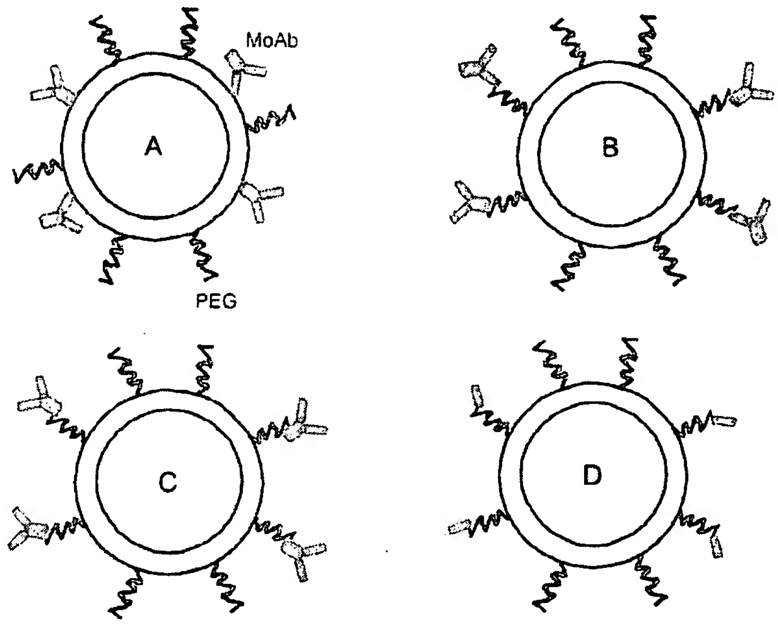

Fig. 1. Overview of the currently used types of PEG–immunoliposomes. The main difference is the position of the antibodies on the PEG–liposomes; they can be attached either directly to the liposomal bilayer (A) or to the distal end of the PEG-chains (B, C and D). In most coupling methods antibodies are randomly derivatized for attachment to functionalized groups on the liposome. This results in a random orientation of the antibody molecules on the liposomal bilayer (A) or on the PEG-chains (B), thereby resulting in exposition of the Fc-portion of at least part of the attached antibodies. One method has been described in which the antibody molecules are specifically attached via their Fc-portions to a hydrazide-group at the terminal end of the PEG-chains (C), resulting in correctly orientated antibodies at the PEG-chains and diminished Fc-exposition [166]. The presence of the Fc-portion can be completely avoided by using Fab′-fragments which can be attached to the terminal end of PEG-chains for example by exploiting the free thiol-group that is created after reducing $F(ab')_2$ fragments into Fab′ (D).

and colleagues who investigated whether the use of Fab′ fragments instead of whole antibodies makes a difference in terms of pharmacokinetics and biodistribution of PEG–immunoliposomes [10,36]. PEG–immunoliposomes exposing per particle approximately 51 whole antibody molecules conjugated at the distal ends of the PEG-chains were found to be rapidly removed from the circulation (60% after 1 h), whereas the same type of PEG–immunoliposomes, bearing per particle an average of 517 Fab′ fragments derived from the same monoclonal antibody, showed a 6-fold longer circulation time [36]. Moreover, accumulation of Fab′-bearing PEG–liposomes directed against the human carcinoembryonic antigen (CEA) in human gastric cancer tumors in nude mice was 2-fold higher compared to the accumulation of PEG–liposomes bearing whole IgG (approximately 20 and 10% of injected dose/g tissue, respectively) and was comparable with the accumulation of non-targeted PEG–liposomes. These results suggest that either the chemical modification of whole antibody molecules needed for coupling to the liposomes or the Fc-portion of antibody molecules mediate an enhanced clearance rate of antibody-targeted PEG–liposomes. Therefore, the use of Fab′ fragments rather than whole antibodies coupled to the distal ends of PEG-chains seems preferable for directing sterically stabilized liposomes to solid tumors. Nevertheless, many investigators still tend to use whole antibodies as targeting devices for PEG–liposomes.

2.1.3. Immunogenicity

It has become clear that the presence of antibodies on the liposome surface can lead to an immune response. Repeated subcutaneous injections of avidin–liposomes bearing biotinylated murine antibodies elicited isotype-specific antibody responses

against the liposome-associated antibodies in mice [37]. Antibodies against phospholipids were not raised. Another study evaluating the immunogenicity of immunoliposomes showed that repeated i.v. injections of PEG–immunoliposomes bearing antibodies coupled to the distal ends of PEG-chains via a hydrazide linker elicited antibodies with specificity for the Fc-portion of liposome-conjugated antibodies [38]. This immunogenicity resulted in drastically decreased circulation times of liposomes in case of repeated administration. Remarkably, it was demonstrated that antibodies coupled to the liposomes are more immunogenic than antibodies given in free form [38]. This suggests that either the membrane-associated presentation of antibodies or the chemical modification of the antibodies needed for coupling to the liposomes accounts for the enhanced immunogenicity. Immunogenicity occurring when murine antibodies are used for liposome-targeting may become less of a problem when smaller antibody fragments (like Fab′ or scFv) and humanized antibodies are used. Nevertheless, immunogenicity is an important factor to take into account when developing immunoliposomal drug carriers for cancer therapy and deserves more attention in future studies.

2.1.4. Passage of the vascular endothelium

Immunoliposomes need to cross the tumor vasculature in order to reach tumor cells located outside the blood compartment. Normal vascular endothelium consists of a continuous lining of endothelial cells, which are tightly connected with each other by tight junctions. Beneath this cellular layer is the basement membrane and in larger vessels, an additional layer of smooth muscle cells is present. This tight barrier prevents the passage of large molecules and particulates. At certain sites in the body, such as liver and spleen, the vascular anatomy is different. At these sites, the endothelial lining contains fenestrations that are approximately 80 to 120 nm in diameter. In solid tumors the vessels formed by the process of angiogenesis often show an increased permeability due to large fenestrae (up to 400 nm) and an irregularly formed basement membrane [39,40]. At tumor sites with an increased vascular permeability, liposomes can extravasate, provided that they are small enough and circulate long enough [41–43]. It has also been suggested that liposomes may extravasate by transcytosis throug cells [44]. However, convincing expe dence that this is really the case is stil

2.1.5. Intra-tumoral transport

Unfortunately, tumors often develop that intra-tumoral penetration of partic interstitium is hindered [45,46]. Ra tumors often have a poorly develo drainage system. This, together with vascular permeability of the neovasc in high interstitial pressure inside tu the convective transport of extrava towards the tumor core. Moreover, th genesis process may yield vessels w tural defects, such vessels showing meability are not equally distrib tumor. As a consequence, extravasat heterogeneously distributed throug and are mainly located in peri [42,43].

There has been controversy as to sated PEG–liposomes are taken up macrophages, thus limiting intera cells. A recent study showed th extravasated PEG–liposomes by m the tumor is minor [47]. In contras other reports show perivascular re sated sterically PEG–liposomes tumor-resident macrophages [48 findings may be explained by m ences in tumor architecture and th residing macrophages. Actively that expose the constant regi antibodies may be even more s phage uptake [50]. The use of lacking the constant part (like F helpful in reducing the uptake by tumor-associated macrophag

The binding site barrier hyp Weinstein et al. [52] for anti immunoliposomes will not pen tumor but bind to the first targe In case of solid tumors, the countered by i.v. administer will be those located directly lining (perivascular zone). A non-targeted long circulating

have shown that with increasing PEG content and increasing PEG size, the coupling efficiency of antibodies to the bilayer of liposomes decreases [8,9]. Furthermore, the interaction of antibodies coupled to the bilayer of PEG-grafted liposomes with their target antigens may be hampered due to steric hindrance caused by the long PEG chains [59–62]. Thus, although PEG–liposomes with antibodies coupled directly to the liposome surface show circulation times comparable to non-targeted PEG–liposomes [63,58] the targeting efficiency can be reduced by the presence of long PEG chains on the surface of these liposomes. This problem of steric hindrance has been successfully solved by coupling the antibodies or antibody fragments to the distal end of functionalized PEG–lipids [64,65]. Such PEG–immunoliposomes combine active targeting capacity with long circulation properties.

Antibody orientation is a major factor influencing the target cell binding of PEG–immunoliposomes. As in most coupling methods the antibodies are randomly derivatized for achieving attachment to functionalized groups on liposomes, part of the antibodies will be attached to the liposomes via their antigen-binding domain resulting in loss of target receptor-binding capacity of at least part of the attached antibodies (Fig. 1). Methods which result in specific orientation of either whole antibodies or Fab′ fragments exist [9,66] and are preferred for optimal binding.

2.2.2. *Therapeutic availability*

After tumor cell binding, the encapsulated drug should become therapeutically available. In principle, the delivery of encapsulated compounds to tumor cells can take place via four different mechanisms: (1) release of encapsulated compounds from cell surface-bound immunoliposomes with subsequent uptake of free drug by the tumor cells (2) transfer of lipophilic drugs from the immunoliposomal bilayers to the plasma membrane of tumor cells (3) endocytosis of cell-surface receptor-bound immunoliposomes with subsequent intracellular release of encapsulated compounds and (4) fusion of the immunoliposomal membrane with the target cell membrane or endosomal membrane. The first mechanism aims for extracellular release of liposome-encapsulated compounds, whereas in the latter three mechanisms, the drug will be released onto or inside the cell. Intracellular release of antitumor drug has as main advantage that it may overcome multidrug resistance [67–69]. In general, tumor cells circulating in the bloodstream require intracellular delivery as extracellular delivery will result in fast diffusion and redistribution of the drug over the blood compartment [70]. In the case of solid tumors, the extracellular release of drug at the tumor site seems preferable as this may lead to diffusion of drug within the tumor mass allowing the drug to also reach those tumor cells that do not express the targeted antigens or that are out of reach for the relatively large immunoliposome carriers. This so-called 'bystander' effect may also occur after intracellular delivery of certain drugs which have physicochemical characteristics that promote the leakage or active transport of a fraction of these drugs out of the target cells.

2.2.2.1. *Extracellular release of encapsulated drug from surface-bound immunoliposomes*

Drug may be released from immunoliposomes which are bound to the surface of tumor cells. The major advantage of this mechanism of delivery is that it does not require that immunoliposomes bind to all tumor cells. The distribution of free drug throughout the tumor will enhance the bystander effect to those tumor cells that do not have any immunoliposomes bound to their surface. In this case, immunoliposomes act as an intra-tumoral drug depot from which free drug is slowly released and taken up by target cells as well as non-target cells within a solid tumor. For effective delivery, immunoliposomes should retain the encapsulated drug while circulating and efficiently release the drug after tumor cell binding. The recent discovery that PEG-modified lipids can undergo spontaneous transfer between membranes and the development of cleavable PEG–lipids [71] provide the technical tools to develop PEG–immunoliposomes that destabilize in time due to slow shedding of the PEG coat. Based on this PEG-shedding mechanism, liposomes have been developed that, after loss of PEG-modified lipids, become highly fusogenic and unstable [57]. In addition, target-sensitive immunoliposomes have been constructed that were composed of dioleoylphosphatidylethanolamine (DOPE) (80%),

dioleoylphosphatidic acid (DOPA) (20%), and a small amount of specific antibody conjugated to N-glutaryl-phosphatidylethanolamine. The presence of N-glutaryl-phosphatidylethanolamine equally distributed in the liposomal bilayer stabilizes the lipid bilayer containing high amounts of hexagonal phase-forming DOPE. Upon target cell binding of these target-sensitive immunoliposomes, it is thought that capping of the N-glutaryl-phosphatidylethanolamine-conjugated antibodies results in an unequal distribution of N-glutaryl-phosphatidylethanolamine within the liposome bilayer inducing rapid destabilization [72]. Whether such liposomes are stable during circulation in the bloodstream remains to be investigated.

An external trigger can potentially enhance the release of immunoliposome-encapsulated drugs in close proximity of tumor cells, provided that the immunoliposomes have an appropriate composition. Such an external trigger may be the local heating of a tumor (thermosensitive immunoliposomes) [73–76] change in pH (pH-sensitive immunoliposomes) [55,77] or laser light (photosensitive immunoliposomes) [78–80]. An obvious requirement for such trigger approaches is that the exact location of the tumor within the body must be known. Besides, the released drug should be able to enter the target cell on its own, as most anticancer agents exert their function inside the cell.

2.2.2.2. Selective transfer of lipophilic (pro)drugs from immunoliposomes to tumor cells

Immunoliposomes are an attractive carrier system for the delivery of lipophilic anticancer drugs or prodrugs to tumor cells. The liposomal bilayer may be exploited for incorporation of either lipophilic drugs or lipophilic derivatives (prodrugs) of hydrophilic drugs that are not efficiently retained in the aqueous phase of the liposome. Examples of the latter are arabinofuranosylcytosine (Ara-C) [81], 5-fluorouracil (5-FU) [82], 5-fluoro-2′-deoxyuridine (FUdR) [83,84] and methotrexate [85]. To date, only a few studies have been published regarding the incorporation of these lipophilic drugs into immunoliposomes for specific targeting [86–88]. One example of lipophilic drug incorporation in immunoliposomes will be further discussed as this shows a unique way by which lipophilic drugs can enter tumor cells. FUdR, which as a free substance is used in the clinic for the treatment of liver metastases of colon cancer, shows rapid leakage after encapsulation in liposomes. Therefore a lipophilic dipalmitoyl prodrug (FUdR-dP) was developed which remains firmly associated with the liposomes, also under in vivo conditions [84,89]. FUdR-dP incorporated in long circulating immunoliposomes targeted to lung endothelial cells, resulted in increased antitumor activity towards lung metastatic growth of mouse mammary carcinoma [86]. More recently, FUdR-dP incorporated in tumor cell targeted immunoliposomes showed a more than 10-fold increased antiproliferative activity in vitro towards rat CC531 colon cancer cells as compared to drug-containing liposomes without antibody [88]. Although these immunoliposomes bind to a high extent to the target cells, they are not internalized. However, the cells rapidly hydrolyze the FUdR-dP yielding the active drug intracellularly. Inhibition of endocytic uptake and intralysosomal degradation resulted in a major inhibition of the hydrolysis of the prodrug, demonstrating that the hydrolysis takes place intracellularly [90]. These results led to the postulation of a selective transfer mechanism for immunoliposomal FUdR-dP (Fig. 2). During the high-affinity interaction of the immunoliposomes with the tumor cells the FUdR-dP is selectively transferred from the immunoliposomal bilayer to the plasma membrane (step 1). From there the drug is transferred into the cell by constitutive pinocytic and endocytic processes (step 2) and hydrolyzed intra-lysosomally (step 3). Subsequently, the hydrolyzed drug diffuses into the cytoplasm (step 4) from where it is either transferred to the nucleus exerting its cytotoxic effect (step 5) or released into the extracellular compartment (step 6), where it can have cytotoxic effects on other tumor cells (bystander effect). An important message to be learnt from these studies is that immunoliposomes can mediate efficient intracellular delivery of a lipophilic drug without being internalized. We believe that this advantage may be more generally applicable for lipophilic (pro)drugs incorporated in immunoliposomes.

2.2.2.3. Internalization of surface-bound immunoliposomes

Certain proteins that bind to cell-surface receptors are rapidly internalized by the cells before they can dissociate from their receptors. This process, which

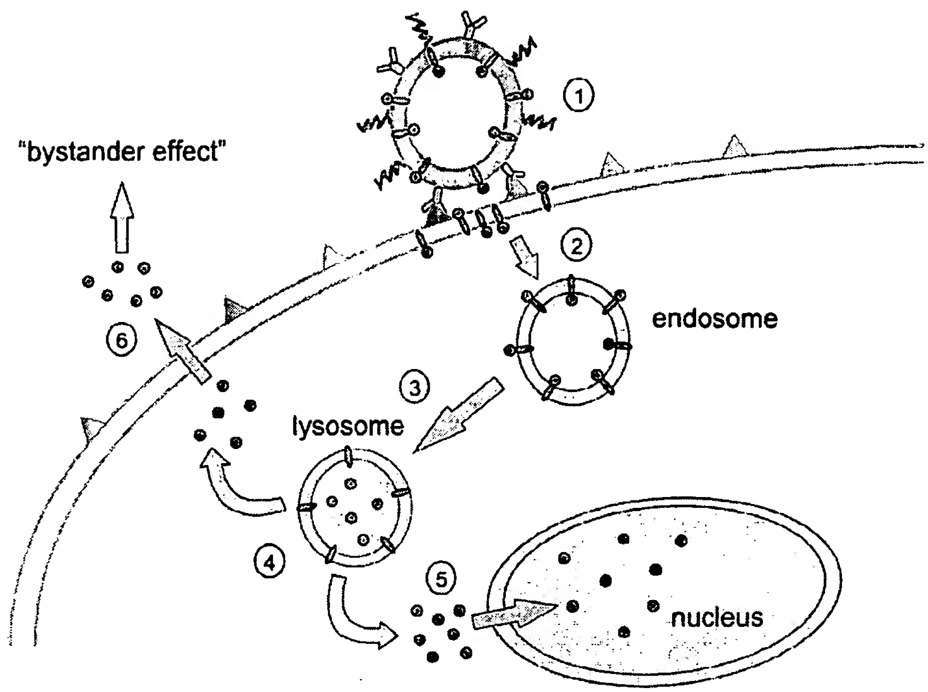

Fig. 2. Schematic presentation of a selective transfer model proposed for a lipophilic dipalmitoyl prodrug of the anticancer agent FUdR from immunoliposomes to the plasma membrane of tumor cells. After target cell binding, the immunoliposome incorporated FUdR-dP is transferred to the plasma membrane of the tumor cell (1). The prodrug is internalized by constitutive endocytic or pinocytic processes (2) and hydrolyzed intra-lysosomally (3). The active drug FUdR then diffuses into the cytoplasm (4) from where it is either transferred into the site of action, the nucleus (5) or released extracellularly (6) where it can exert a cytotoxic effect on surrounding tumor cells (bystander effect). Adapted from Scherphof et al. [50].

is called receptor-mediated endocytosis, is recognized as an important and general mechanism by which cells take up nutritional and regulatory proteins from extracellular fluid. This internalization process can also be utilized for the cellular uptake of immunoliposomes. A number of reports have demonstrated that small immunoliposomes targeted to certain cell-surface receptors (e.g. epidermal growth factor receptor, transferrin receptor, folate receptor) are internalized in a way that closely resembles receptor-mediated endocytosis [13,35,59,91]. Unfortunately, this route of entry does not guarantee full therapeutic availability of internalized drug molecules. Many therapeutic agents act at an intracellular target site to be reached via the cytosol. Immunoliposomes internalized by the mechanism of receptor-mediated endocytosis will eventually end up in lysosomes where they are degraded together with the encapsulated drug molecules. The physicochemical nature of the encapsulated drug may be compatible with the need to escape from endosomal or lysosomal compartment [92]. Molecules like doxorubicin [93] may be resistant to the action of lysosomal enzymes and/or low pH and able to cross the endosomal or lysosomal membranes. Also, lipophilic drugs may transfer from the liposomal membrane to the endosomal membrane during endocytosis via the mechanism described before (Section 2.2.2.2). Endosomal escape of hydrophilic drugs into the cytosol may be promoted by the incorporation of pH-dependent fusogenic properties into the liposomal carrier. These pH-dependent fusion mechanisms will be dealt with in Section 2.2.2.4.

2.2.2.4. Fusion with the plasma membrane of target cells

Fusion of immunoliposomes with cells is an attractive strategy to actively deliver entrapped drug molecules into the cytosol. In principle, there are two routes by which cytosolic delivery via fusion can be

achieved (1) fusion of immunoliposomes with the plasma membrane of the target cell initiated after the immunoliposomes have bound to the target cells, or (2) pH-dependent fusion of immunoliposomes with the endosomal membrane, after being internalized by the target cell via receptor-mediated endocytosis.

Fusion of surface-bound immunoliposomes with the plasma membrane of the target cell has two major advantages. First, it does not require internalization of the immunoliposomes and consequently there is no need to target to an internalizing epitope, which increases the number of potential target epitopes for immunoliposomes. Moreover, plasma membrane fusion does not add a restriction to the size of fusogenic immunoliposomes in contrast to immunoliposomes that are designed for internalization. Second, fusion of immunoliposomes with the plasma membrane results in release of encapsulated drug directly into the cytosol. However, it should be realized that immunoliposomes with fusogenic activity at neutral pH may also fuse with non-target cells as a consequence of aspecific adherence. In addition, unwanted inter-liposomal fusion events may occur.

In contrast to fusion of immunoliposomes directly with the plasma membrane, fusion with the endosomal or lysosomal membranes requires internalization. To this end, immunoliposomes should be targeted to receptors with known internalizing properties. In addition, the size of liposomes should not hamper endocytosis and should therefore be small (preferably < 100 nm) [94]. pH-sensitive liposomes have been used for the cytosolic delivery of encapsulated drug via the pathway of receptor-mediated endocytosis [77]. Liposomes with pH-dependent fusogenic properties can be constructed from dioleoylphosphatidylethanolamine (DOPE) in combination with acidic lipids, such as N-palmitoylhomocysteine (PHC), cholesterylhemisuccinate (CHEMS), oleic acid (OA) or diacylsuccinylglycerols. Under physiological conditions, DOPE preferentially forms inverted hexagonal phase (H_{II}) rather than bilayers. However, admixing DOPE with one of the acidic lipids mentioned above prevents H_{II} phase formation and yields stable bilayers at neutral pH. Upon acidic exposure, however, protonation of the acidic lipids occurs thereby neutralizing their negative charges and inducing destabilization of the liposomal bilayers and/or induction of pH-dependent lipid mixing [95–98]. Another strategy to achieve pH-dependent membrane destabilization or fusion is to incorporate pH-sensitive polyethylene glycol derivatives having carboxyl groups [99]. It was demonstrated that succinylated poly(glycidol) (SucPG) coupled to egg yolk phosphatidylcholine (PC) liposomes as a fusogenic polymer induced pH-dependent lipid mixing of liposomal lipids and yielded cytoplasmatic delivery of encapsulated calcein [99].

Membrane-active peptides can also be used to promote the cytosolic delivery of liposome-encapsulated drug from the endosomal/lysosomal compartment. Such peptides may act by destabilizing membranes, forming pores or inducing membrane fusion (reviewed by Plank et al. [100]). A number of synthetic, amphipatic peptides have been shown to reveal fusogenic activity. For example, the amphipatic 30-residue peptide named GALA mimics the behavior of fusion peptides derived from viral fusion proteins. It was shown that GALA induces fusion of liposomes composed of PC in a pH-dependent fashion [101]. Puyal et al. [102] attached a 14-amino-acid residue GALA-type peptide to liposomes and showed that intermixing of lipid between the liposomes occurs in a pH-dependent fashion. Similar results were obtained with a synthetic fusion peptide that induces fusion at neutral pH when coupled to the liposomal membrane [103,104]. The latter two studies clearly show that membrane-active properties can be grafted onto liposomes in order to enhance the cytosolic delivery of encapsulated compounds. Up to now no reports have been published demonstrating the utility of fusogenic peptide-bearing immunoliposomes for cytosolic delivery enhancement.

3. Immunoliposomes in vivo: current status

In early days, the utility of conventional immunoliposomes in vivo was strongly limited by their rapid clearance by the MPS. With the advent of long-circulating liposomes that are able to oppose uptake by the MPS, targeting of liposomes to tissues other than the MPS is now much more realistic. One of the most critical issues to consider when developing

immunoliposomal systems is whether the surface-attached antibodies can access the tumor cell surface in vivo. It is clear that the route of administration is an important determinant for the degree of in vivo targeting that can be achieved. For this reason, the current status of immunoliposomes as drug carriers in cancer therapy will be discussed separately for immunoliposomal targeting to cells within the vasculature after i.v. administration, to tumor cells residing in the peritoneal cavity after local administration and to solid tumors after i.v. administration.

3.1. Intravenous administration of immunoliposomes to target cells within the vasculature

3.1.1. Circulating tumor cells

Hematological malignancies (e.g. lymphomas, multiple myeloma or leukemias) seem attractive candidates for immunoliposome-based therapy as circulating tumor cells are likely easily accessible targets for i.v. administered immunoliposomes. Lopes de Menezes et al. targeted sterically stabilized immunoliposomes containing doxorubicin (DOX–SSIL) to CD19+ Namalwa cells (human B-cell lymphoma) circulating in the bloodstream of SCID mice. In vitro evidence pointed to internalization of tumor cell-bound anti-CD19 SSIL by receptor-mediated endocytosis [70]. Evaluation of the therapeutic effect in vivo showed a significant increase in survival (77% increase in lifespan) for the tumor-bearing mice treated with anti-CD19 DOX–SSIL in comparison with non-targeted DOX–SSL or free DOX. The effectiveness of treatment was dependent on the dose of inoculated tumor cells and the time interval between tumor cell inoculation and intervention. Best therapeutic results were obtained with early interventions (i.e. 1 h after i.v. injection of tumor cells) when inoculated tumor cells are most likely still circulating. Multiple weekly treatments (days 1, 8 and 15 after tumor inoculation) only slightly improved the survival times of mice, indicating that target cells may have become relatively inaccessible or have grown to a size that hinders effective penetration of DOX–SSIL formulations into the tumor mass. The better therapeutic effect of DOX–SSIL compared to non-targeted DOX–SSL was explained by efficient internalization of CD19-bound DOX–SSIL by Namalwa cells followed by intracellular release of DOX from the internalized liposomes. As non-targeted DOX–SSL were not internalized by the target cells in vitro, the results show the necessity of internalization to increase the efficiency of drug delivery to tumor cells within the vasculature.

Recently, similar results were obtained with DOX-containing sterically stabilized anti-idiotype immunoliposomes in a murine B-cell lymphoma model [105]. Treatment of tumor-bearing mice with DOX containing anti-idiotype SSIL resulted in a significantly greater median survival time as compared to mice treated with either saline, free DOX or plain DOX–SSL. Also in this study it could be shown in vitro that anti-idotype-targeted SSIL were efficiently internalized by the malignant B-cells. Another option to explain the better therapeutic effects of the DOX-anti-idiotype SSIL formulations used in the above mentioned studies, is that SSIL bound to target cells induced the so-called 'target cell dragging phenomenon', as proposed by Peeters and colleagues [106,107]. This phenomenon implies that binding of the immunoliposomes to the circulating target cells induces an opsonic effect leading to rapid uptake of the tumor cells by hepatosplenic macrophages.

A potential clinical hazard related to immunoliposome targeting to circulating cells is the possible occurrence of agglutination. Circulating tumor cells may be agglutinated due to multivalent interaction of immunoliposomes with more than one tumor cell. Moreover, immunoliposomes may be agglutinated in the blood circulation due to the presence of soluble bi- or multivalent target antigens shed from the surface of either circulating tumor cells or tumor cells within a solid tumor. It was demonstrated that immunoliposomes targeted i.v. to the idiotype of B-cell receptors on malignant B-cells caused clinical signs of dyspnea, lateral recumbancy, peripheral limb cyanosis, convulsion and occasional death in tumor-bearing mice [108]. These clinical symptoms were the result of agglutination of circulating immunoliposomes by soluble antibodies produced by the malignant B-cells which causes occlusion of small capillaries as the i.v. injection of purified antibodies from these malignant B cells after administration of immunoliposomes gave the same symptoms. The above discussed study on the therapeutic efficacy of

CD19 DOX–SSIL in SCID mice bearing CD19+ human B-cell lymphomas did not report any of these symptoms.

3.1.2. Endothelial cells

Endothelial cells lining the vasculature represent an easily accessible target to i.v. administered immunoliposomes. Maruyama and colleagues have targeted SSIL to lung endothelial cells by conjugating mAb 34A to the distal ends of liposome-attached PEG-molecules [10,64,109]. This mAb is specific for thrombomodulin which is expressed at high levels on mouse lung endothelial cells. In Balb/c mice up to 60% of i.v.-injected 34A-SSIL was found in the lung within 15 min of injection. Liposomes without conjugated antibodies or with irrelevant antibodies conjugated to the PEG-chains did not accumulate in the lung ($<0.4\%$ of injected dose) but were mainly found in the liver (50% of injected dose). The excellent targeting efficiency of 34A-SSIL has been utilized to achieve high levels of antineoplastic drugs in lungs of mice bearing metastasized tumors [86]. It was shown that mice treated with FUdR-dP incorporated into 34A-immunoliposomes yielded a significant increase in lifespan compared to mice treated with FUdR-dP dispersed in a lipid emulsion or incorporated in non-targeted liposomes. This study indicates that an indirect targeting approach (i.e. active targeting of immunoliposomal antineoplastic drugs to cells other than the tumor cells) may be attractive to obtain high levels of drug in close proximity of tumor cells by targeting to readily accessible targets. The lipophilic characteristic of the FUdR-dP was proposed to play a role in the trans-endothelial delivery of the drug.

3.2. Local administration of immunoliposomes to target tumors confined to the peritoneal cavity

Tumor cells located in the peritoneal cavity present another accessible target for immunoliposomes if the immunoliposomes are injected intraperitoneally (i.p.). This is exemplified by studies on the i.p. administration of immunoliposomes for the treatment of ovarian carcinoma [87,110,111]. Straubinger et al. attached whole antibodies directed against CA-125 carcinoma antigen to the surface of liposomes [111] and showed that i.p. injected OC-125 immunoliposomes were able to bind to ovarian cancer cells (OVCAR-3) growing as an ascitic tumor in the peritoneal cavity of nude mice.

In another study on ovarian carcinoma treatment, the lipophilic prodrug 5′-palmitoyl-5-fluorouridine (PF) was incorporated into immunoliposomes [87]. The investigators utilized whole antibodies directed against the CAR-3 antigen, which is abundantly expressed on human ovarian carcinoma cells and other adenocarcinomas. Intraperitoneal injection of anti-CAR-3 immunoliposomes into the OVCAR-3 xenograft model resulted in a drastic reduction of the tumor mass. The PF–immunoliposomes were four times more effective than the corresponding non-targeted PF–liposomes and 18 times more effective than the free drug, illustrating the superiority of PF–immunoliposomes.

Nässander et al. used immunoliposomes bearing covalently coupled Fab′ fragments of the monoclonal antibody OV-TL3, which is directed against the antigen OA3 present on more than 90% of all human ovarian carcinoma cells [112]. When these immunoliposomes were administered i.p. in the OVCAR-3 xenograft model, a high fraction (about 70%) of the injected dose was bound to the tumor cells within 30 min. Encouraged by these excellent in vivo targeting results, doxorubicin (DOX) was loaded into these immunoliposomes (IL) and evaluated in the same i.p. xenograft model [110,113]. Although the DOX-IL formulations had a superior antitumor effect compared to non-targeted DOX–liposomes in vitro, no difference in antitumor activity between the targeted and non-targeted formulation was observed. As no evidence for cellular internalization of cell-bound immunoliposomes was obtained, a similar degree of premature drug leakage from both the targeted and non-targeted DOX–liposomes within the peritoneal cavity was suggested to underlie the antitumor effects seen. The lack of therapeutic benefit of Fab′-targeted liposomes clearly illustrates the point that even well-targeted immunoliposomes can fail due to lack of cellular internalization and/or uncontrolled drug release patterns.

3.3. Intravenous administration to target solid tumors and metastases

Liposome investigators have been able to exploit the abnormal blood vessel structures associated with tumor angiogenesis for extravasation of liposomes

[46]. As discussed in Section 2.1.4, it is now well documented that immunoliposomes must both be small and exhibit long circulation times to achieve preferential localization within solid tumors [43,49]. Active targeting of sterically stabilized liposomes (SSL) by conjugating antibodies or antibody fragments to the liposomal surface may further improve the therapeutic efficacy by increasing the specific uptake of liposomal drug formulations into tumor cells within a solid tumor. However, one should realize that the attachment of antibodies or antibody fragments to the surface of SSL and especially to the distal ends of PEG–lipids on SSL may oppose the MPS-avoiding capacities of SSL (see Section 2.1.2) [8,36].

Maruyama and colleagues have evaluated the efficiency of tumor targeting of SSIL in a xenograft model of human gastric cancer [10,36]. SSIL were constructed by conjugating either whole antibodies or Fab′ fragments of anti-CEA mAb 21B2 to the distal ends of the PEG-chains. The tumor localization of these SSIL 24 h after i.v. injection were compared with non-targeted SSL and bare liposomes (i.e. without any surface-bound molecules). It was shown that the most efficient tumor localization was obtained with non-targeted SSL or Fab′-targeted SSIL (both 20% of injected dose/g tumor). Whole antibody-targeted SSIL localized substantially less at the tumor site (10% of injected dose/g tumor) but tumor accumulation was still higher than for bare liposomes (7% of injected dose/g tumor).

Reports demonstrating an improved antitumor efficacy of antitumor agents encapsulated in actively targeted SSIL relative to non-targeted SSL are scarce. Ahmad et al. [114] studied immunoliposomes with avidin covalently coupled to the bilayer of PEG–liposomes. Biotinylated monoclonal antibodies that recognize specific epitopes on the surface of lung squamous carcinoma cells avidly bound to these avidin-bearing liposomes. Such 'two-step-targeted' immunoliposomes were loaded with doxorubicin (DOX) and subsequently injected i.v. in mice bearing murine squamous carcinoma cells in the lungs. Treatment with DOX–SSIL resulted in significantly improved antitumor activity compared to treatment with free DOX or non-targeted DOX–liposomes. The success of this approach was ascribed to efficient targeting to tumor cells in combination with the release of DOX from tumor cell-bound immunoliposomes and subsequent uptake of free DOX by tumor cells. Regression of more advanced tumors did not occur, probably a result of poor tumor penetration or down-regulation/shedding of the target epitope.

Kirpotin at al. [115] treated HER2/neu over-expressing human breast cancer cells in vitro with DOX-containing immunoliposomes bearing Fab′ fragments of a humanized mAb directed against the HER2/neu antigen. The Fab′ fragments were either attached to the liposomal surface via a short spacer or to the distal ends of PEG-chains. The in vitro tests showed that the immunoliposomes with the Fab′ fragments attached to the distal end of PEG-chains are able to avidly bind to the HER2/neu over-expressing cells and are rapidly internalized via the coated pit pathway. The DOX-loaded anti-HER2/neu SSIL produces significantly increased antitumor activity as compared to free DOX or DOX-loaded non-targeted liposomes (SSL) in xenograft models of HER2/neu-overexpressing breast cancer. Interestingly, the degree of localization of anti-HER2/neu SSIL in HER2/neu-overexpressing tumor xenografts was not increased over that of non-targeted SSL. However, the use of colloidal gold-containing SSIL revealed that the SSIL were distributed throughout the tumor interstitial spaces as well as localized within tumor cells [116]. In contrast, nontargeted SSL were located predominantly within tumor-associated macrophages. Thus, increased deposition in the inter-cellular spaces within the tumor tissue outside tumor-associated macrophages, as well as the intracellular delivery of DOX may have contributed to the superior antitumor activity of DOX-loaded anti-HER2/neu SSIL. Therapeutic superiority of targeted SSIL was not observed by Goren et al. [117]. The latter group also studied the in vivo antitumor effects of DOX-loaded anti-HER2/neu SSIL and concluded that the antitumor activity of targeted and non-targeted DOX–SSL was similar. Whether this discrepancy in outcome is related to differences in tumor model or to the fact that Goren et al. utilized complete mouse antibody rather than humanized Fab′ is not clear.

In another study, mice bearing human ovarian cancer xenografts were treated with either free DOX, DOX–SSL or DOX–SSIL (targeted with mAb B43.13 to human ovarian cancer cells) [53]. Surprisingly, the non-targeted DOX–SSL formulation was

more effective than DOX–SSIL in reducing the rate of tumor growth. This finding was ascribed to the binding site barrier phenomenon (see Section 2.1.5)

4. Recent advances

A general trend that can be deduced from the previous section is that, despite successful results in vitro, the results in vivo are somewhat disappointing: only a few examples of successful anticancer applications of immunoliposomes in vivo exist. Clearly, the immunoliposomal system needs further improvement in order to obtain an effective drug delivery vehicle for in vivo application. Several major problems have been identified. Recent developments to tackle these problems are discussed below.

4.1. Development of alternative homing devices

4.1.1. Antibody-based homing devices

Studies with antibody-targeted PEG–liposomes have shown that a high density of antibodies attached to the distal ends of the PEG chains evoke antibody-mediated recognition and enhanced clearance by the RES [8]. The mechanism of clearance is thought to be — at least in part — Fc-receptor-mediated as the coupling of Fab′ fragments did not induce enhanced clearance at similar high densities. Therefore, the use of Fab′ fragments rather than whole antibodies seems advantageous [10,118]. The presence of antibody molecules on the liposomal surface can also induce an immune response. Studies by Phillips et al. and Harding et al. show that upon repeated injections of immunoliposomes in mice or rat, isotype-specific antibodies were generated against the antibodies conjugated to the liposomes [37,38]. Likely, immunogenicity problems encountered with antibody therapy in humans, such as human anti-mouse antibody (HAMA) responses [119] will also hold for immunoliposomal drug formulations. Immune responses may severely hinder repeated dosing regimens for immunoliposomes in the clinic. In order to prevent HAMA responses, humanized mAbs have been produced, in which the complementary determining regions (CDRs) of murine mAbs have been grafted onto human antibodies [120,121]. Although such chimeric antibodies largely consist of regions of human origin, still an antibody response may be induced against the grafted variable regions of murine origin. A better option is the use of fully human antibodies, which has become a possibility with the development of transgenic mice that produce antibodies of human origin [122,123]. These mice may provide the optimal source for immunotolerant antibodies for targeted drug delivery in humans. However, one should keep in mind that not only the origin of antibodies determines its antigenicity, also coupling chemistry involving the chemical modification of antibodies and the use of anchoring molecules may introduce immunogenicity problems.

Besides whole antibody molecules and Fab′ fragments, smaller antibody fragments have been studied. Lipid-tagged human single chain Fv antibody fragments (scFv) have been constructed and successfully incorporated into liposomes [124,125] and used to target liposomes to B-lymphocytes in vitro [125]. Theoretically, there are several advantages for using lipid-tagged scFvs over whole antibodies for liposome targeting: (1) The smaller size of scFv antibodies together with their human origin may make immunoliposomes less immunogenic than whole antibodies. (2) The absence of an immunoglobulin constant region will prevent rapid Fc-receptor mediated clearance of scFv-targeted liposomes. (3) There is no need to chemically modify the lipid-tagged scFv-antibodies for coupling to liposomes. They will spontaneously insert into the phospholipid bilayers due to the presence of the lipid tag. Potential loss of binding affinity and/or induction of immunogenicity due to chemical modification procedures are therefore avoided. (4) The availability of large phage display libraries of human scFv-fragments, the possibility of large scale production, and the simple selection procedure of antigen-specific scFv-fragments favour the use of scFv fragments instead of murine antibodies. However, in vivo studies utilizing scFv-immunoliposomes are lacking at present. Therefore, issues like immunogenicity and stable insertion of the lipid tag into the bilayer are not addressed yet.

4.1.2. Oligonucleotide aptamers

Recently, a novel method for the production of antigen binding molecules has been developed. This method makes use of oligonucleotide aptamers that in their specific three-dimensional conformation can

bind to proteins with specificities and affinities comparable to antibody molecules. Aptamers can be selected from large libraries of nucleic acid sequences with the systemic evolution of ligands by the exponential enrichment (SELEX) method [126–128]. From such libraries ($>10^{15}$ different aptamers) aptamers to virtually any protein can be selected and potentially be used as homing devices to target liposomes [16].

4.1.3. Other ligands

Naturally occurring ligands to cell-surface receptors such as vitamins, hormones and growth factors may also be used as immunotolerant homing devices. They offer some potential advantages over antibodies, such as lack of immunogenicity, high affinity for their receptors, and low cost. It was shown that liposomes to which the epidermal growth factor (EGF) was covalently coupled could be targeted to hepatocytes expressing the EGF-receptor [13]. As many carcinoma cells show a relatively high expression of the EGF-receptor, EGF-targeted liposomes may be useful for tumor targeting [12,13].

Folate has also been studied as targeting ligand [11,129]. The folate receptor has been identified as a surface marker in ovarian carcinomas. Receptor overexpression is found in many other types of cancer as well. Folate-targeted liposomes that specifically bind to cells that overexpress the folate receptor have been designed [130]. When folate is directly attached to the phospholipid headgroups or when a short spacer is used to couple folate with the bilayer, the folate-exposing liposomes are not recognized by the folate receptor. A long PEG spacer (M_r 3350) between the folate and the liposome surface is required for receptor recognition. Such folate-targeted liposomes are extensively internalized by receptor-mediated endocytosis after target cell binding. Incorporation of DOX resulted in 86-fold higher cytotoxicity to folate receptor-expressing tumor cells compared to non-targeted liposomes and 2.7 fold higher cytotoxicity relative to free DOX [131].

4.2. Improving the therapeutic availability of immunoliposomal drugs

4.2.1. Thermosensitive immunoliposomes

Evidence that the therapeutic efficacy of liposomal drugs can be drastically improved by enhancing the drug release from liposomes extravasated into tumor tissue was elegantly demonstrated with thermosensitive liposomes [73–76,132,133]. The heat-induced drug release concept is based on the large increase in permeability of liposomal bilayers around their phase transition temperature. Local heating of tumor tissue up to this phase transition temperature will enhance drug release out of liposomes present in the heated area [75]. In vivo fluorescence videomicroscopy has been used to study the extravasation of thermosensitive liposomes and the release of their contents in a rat skin flap window chamber which contained a well-vascularized mammary adenocarcinoma [73]. Extravasation and contents release were measured at different temperatures (34, 42 and 45°C). At hyperthermic conditions both the degree of extravasation as well as the rate of drug release from thermosensitive liposomes (composed of DPPC, HSPC, CHOL, PEG-PE) were substantially increased. Improved therapeutic efficacy of thermosensitive liposomes in combination with local heat application has been demonstrated in several studies [74,134,135]. The above mentioned studies all used non-targeted thermosensitive liposomes. Only one study with thermosensitive immunoliposomes in vitro has been reported [76]. The in vivo applicability of thermosensitive immunoliposomes as targeted drug vehicles in cancer therapy remains to be investigated.

4.2.2. pH-Sensitive immunoliposomes

pH is another factor that may be utilized to trigger the release of drugs from immunoliposomes. pH-sensitive immunoliposomes have been prepared that became highly unstable and fusogenic at acidic pH [77,97,136]. pH-sensitive immunoliposomes targeted to internalizing receptors will end up in endosomes where acidification will trigger liposome destabilization and possibly fusion with the endosomal membrane. They have been applied in vitro for the delivery of antitumor drugs into the cytoplasm of tumor cells [137,138]. Indeed it was demonstrated that pH-sensitivity strongly increases the potency of immunoliposomal antitumor drugs. pH-sensitive immunoliposomes have never been tested for in vivo drug delivery to tumors, probably because of evidence pointing to instability in the circulation [139]. The recent discovery that PEGylation of pH-sensitive liposomes improves the stability of these liposomes

in vivo may revive the interest of using pH-sensitive immunoliposomes as antitumor drug carriers in cancer therapy [140].

4.2.3. Utilization of membrane-active peptides

Membrane-active peptides are short, amphipatic peptides containing hydrophobic amino acid sequences. These sequences have in common that they can fold into specific structures (e.g. α-helix, β-sheet) dependent on environmental conditions like pH or the presence of cell membranes. In nature such peptide stretches are efficiently utilized in many biological events involving membrane interactions [100]. Many of these membrane-active peptides have been synthesized and tested for membrane-destabilizing and/or fusogenic properties [141–145]. They show membrane destabilizing activities either at low pH [101,102,146,147] or at neutral pH [103,104,143,148]. Certain membrane-active peptides have fusogenic properties as they can induce the mixing of lipids between distinct liposome particles. Several studies have shown the possibility to couple membrane-active peptides to the bilayer of liposomes [102,103,145]; in some cases an increase in membrane activity of the peptide could be observed after coupling to the bilayer [102,103]. The feasibility of the use of pH-dependent, membrane-active peptides coupled to the surface of immunoliposomes to enhance the cytosolic delivery of anticancer drugs to tumor cells is currently under investigation.

4.2.4. Immuno-enzymosome approach

A totally different approach to obtain high concentrations of therapeutically available drug at tumor sites is the use of immunoliposomes for antibody directed enzyme prodrug therapy (ADEPT) [149,150]. ADEPT was originally described for antibodies [151] but has been adapted for the use of immunoliposomes. Instead of entrapping drug molecules inside immunoliposomes, the immunoliposomes carry anticancer prodrug-activating enzyme molecules on their surface (immuno-enzymosomes). In this two-step approach, the first step involves the targeting of the immuno-enzymosomes to the tumor site. Subsequently, an anticancer prodrug matched with the enzyme is given, which will then be converted to its cytotoxic parent compound selectively near the tumor cell surface, thereby inducing an antitumor effect without systemic toxicity. Recently, we have coupled the enzyme β-glucuronidase, capable of activating anthracycline-prodrugs, to the external surface of immunoliposomes directed towards ovarian carcinoma cells [150,152]. It was demonstrated that after tumor cell binding and subsequent addition of prodrug, the immuno-enzymosomes were able to convert almost all prodrug added resulting in killing of ovarian carcinoma cells in vitro whereas enzymosomes (bearing no specific antitumor antibodies) or immunoliposomes (bearing no enzymes) were clearly ineffective. These findings point to the potential usefulness of immuno-enzymosomes in prodrug activation therapy. The immuno-enzymosome approach is a conceptually attractive strategy as it circumvents the problem of insufficient therapeutic availability of the encapsulated drug contents at the target cell surface. The bystander effect described above (Section 2.1.5) may promote the killing of those tumor cells that are not reached by the immuno-enzymosomes. The possible advantages that immuno-enzymosomes offer over antibody–enzyme conjugates usually used in ADEPT are that (1) one carrier unit of immuno-enzymosomes delivers much more enzyme than one antibody unit, and (2) multivalent interaction of immunoliposomes with target cells may result in higher avidity to the target epitopes as compared to antibodies. Currently, in vivo studies in our group are focused on the i.p. administration of immuno-enzymosomes for the therapy of ovarian cancer micrometastases localized in the peritoneal cavity. It is obvious that i.v. administration of immuno-enzymosomes to target solid tumors will require steric stabilization of their surfaces to prolong circulation times.

4.3. Multiple layer liposomes

As mentioned before, immunoliposomes designed for in vivo cancer therapy should be able to retain drug while in the circulation, but upon interaction with the target cells, they should efficiently release the drug. Ironically, those liposomal characteristics needed to retain the drug in circulation, may counteract the efficient release of drug after binding to the target cell. Several paradoxal requirements should be

combined into one immunoliposomal carrier system allowing immunoliposomes (1) to retain drug completely following intravenous administration (2) to avoid rapid clearance by the MPS and allow extravasation (3) to bind to tumor cells and (4) to efficiently release the entrapped drug after binding to the tumor cells. As these attributes are not needed all at the same time, it may be possible to construct liposomes with different layers in which each layer fulfills one or more of the above mentioned functions (Fig. 3). After performing its function a layer should be shed from the liposomes in order to expose the next layer. Such transformable liposomes or 'multiple layer' liposomes have recently been proposed by Bally et al. [153]. The feasibility of such 'multiple layer' liposomes has been illustrated in a study in which cleavable PEG (thiolytic cleavage) was used to stabilize liposomes containing the non-bilayer forming fusogenic lipid DOPE [71]. The PEG–lipid in this system has a dual function. First, its presence provides steric stabilization to yield prolonged circulation properties and second, it stabilizes the lipid bilayer, thereby preventing DOPE-mediated destabilization of the bilayer and contents release. The study showed that DOPE-mediated membrane destabilization — and as a result release of contents — only occurs upon cleavage of PEG.

The 'multiple layer' liposomes may contain PEG–lipids with different PEG-chain lengths (Fig. 3). For instance, the first outermost layer may consist of PEG-5000 for creating the steric stabilization effects, the second layer of functionalized PEG-2000 for coupling of homing devices for specific binding and subsequent internalization, and the third layer of 'instable' bilayers, stabilized by the two layers of PEG–lipids. Such 'multiple layer' liposomes should shed their layers one by one, starting with the outermost layer. The shedding of the different layers of PEG may be regulated by using different PEG–lipid conjugates. It has been shown that the rate of transfer of PEG–lipids to a large excess of neutral acceptor liposomes is dependent on the length and degree of saturation of the acyl chains present in the lipid anchor [57]. This difference in PEG–lipid transfer can be utilized to control the shedding of PEG-layers from multiple layer liposomes. In addi-

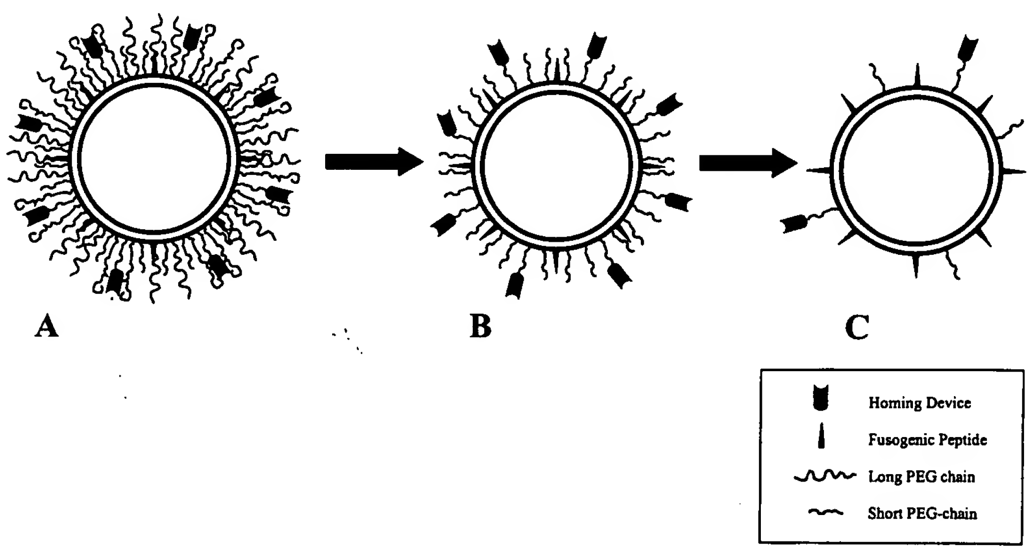

Fig. 3. Schematic representation of 'multiple layer' immunoliposomes at successive stages. Stage A represents a 'multiple layer' immunoliposome while in the circulation. PEG-layers with two different chain lengths preserve the bilayer stability and shield the presence of homing devices (e.g. scFv antibody fragments), thereby assuring long circulation properties. After extravasation and penetration into the tumor interstitium, the outermost PEG-coat should be shed in order to expose the homing devices for tumor cell-binding (stage B). Upon transfer of the innermost layer of PEG–lipids or upon PEG-cleavage, the membrane destabilizing properties of liposomes become activated either by exposure of membrane-active peptides (as shown in stage C) or by hexagonal phase formation of DOPE thereby enabling effective delivery of encapsulated drug.

tion, PEG can be coupled to liposomes by using cleavable bonds with different cleavage kinetics (e.g. acidic or enzymatic hydrolysis) [71,154].

4.4. Targeting to the tumor vasculature

An indirect approach to fight tumor cells with immunoliposomes may be to target the tumor vasculature with the aim of obstructing the blood supply to the tumor (Fig. 4). As tumor cells rely on the continuous supply of nutrition and oxygen like normal cells, depriving the blood supply will result in tumor cell death. As compared to tumor cell targeting, vascular targeting has several attractive features: (1) the endothelial cells are directly accessible for circulating immunoliposome particles. Therefore, tumor cell accessibility problems related to poor extravasation and tumor tissue penetration are avoided. (2) Even the destruction of a small number of endothelial cells may be enough to induce coagulation, resulting in the formation of an occlusive thrombus interfering with tumor perfusion and leading to necrosis. Therefore, tumor vasculature-directed liposomes can be expected to have a greater capacity to induce antitumor effects than liposomes designed for drug delivery to tumor cells. (3) As most solid tumors rely on similar blood vessels for growth, the vascular targeting approach should be

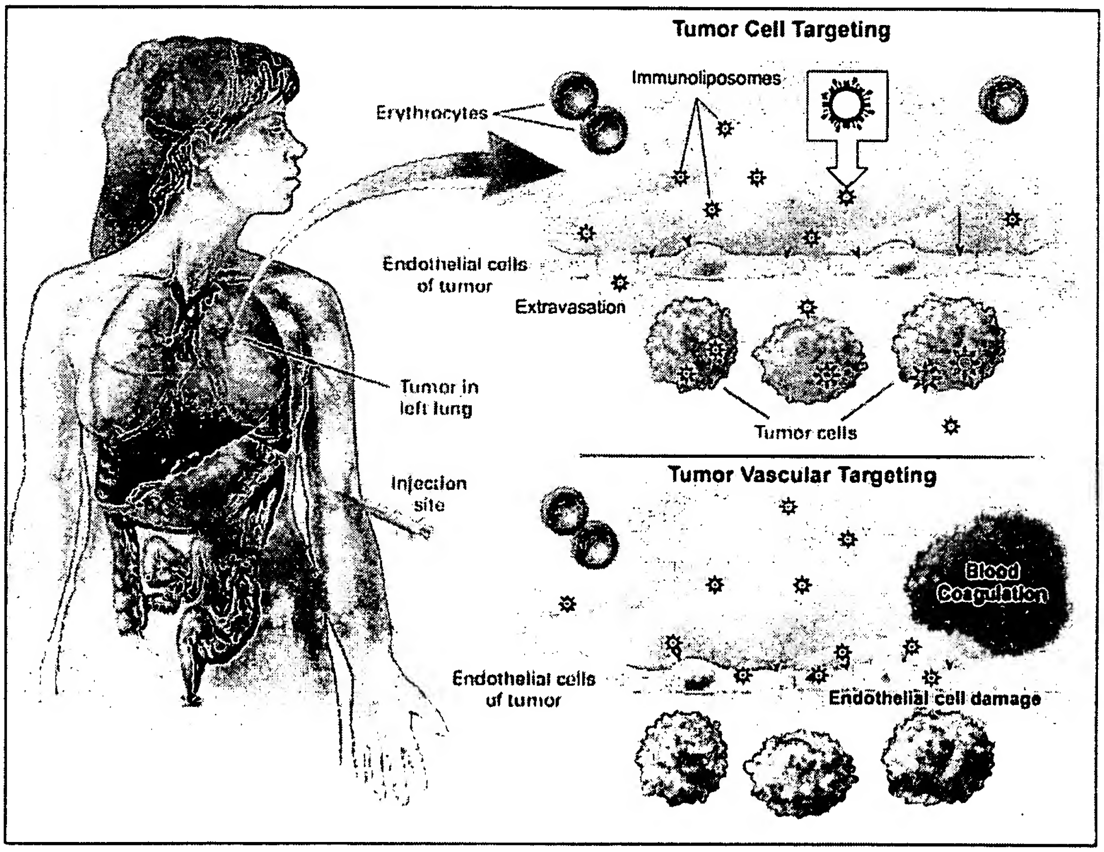

Fig. 4. Concept of tumor vascular targeting versus tumor cell targeting in vivo. Immunoliposomes containing cytotoxic drugs are injected intravenously and travel through the circulation where they can reach tumor vessels. If drug-loaded immunoliposomes are targeted to tumor cells (upper panel), they should extravasate in order to reach and kill the tumor cells behind the tumor vascular endothelial lining. Immunoliposomes targeted to specific receptors on tumor endothelium (e.g. $\alpha V\beta$ integrins or VEGF-receptors) can lead to endothelial cell damage and the induction of blood coagulation (lower panel). In this way, the blood supply to the tumor is obstructed, resulting in massive tumor cell death. Adapted from Schnitzer [167].

applicable to most or possibly all solid tumors. (4) Endothelial cells are genetically stable and therefore expected not to mutate to become resistant to the therapy, as tumor cells often do.

A prerequisite for tumor vascular targeting is of course the existence of highly specific tumor vascular markers. In humans, several cell-surface receptors have recently been identified that are specifically expressed on tumor vascular endothelium which are potentially suitable for tumor vascular targeting purposes [155,156].

The feasibility of killing tumors by attacking their blood supply was demonstrated by several investigators, utilizing tumor vasculature-directed antibody — or peptide conjugates [157–159]. Successful targeting of immunoliposomes to endothelial cells have been shown in vitro [160–163] and in vivo [164,165]. The fact that endothelial cells are well accessible for intravenously injected immunoliposomes is illustrated by work of Maruyama et al. showing targeting efficiencies of immunoliposomes targeted to lung endothelial cells of up to 60% of injected dose [10,109]. However, immunoliposome-based applications of tumor vasculature targeting have not been reported yet.

5. Final remarks

We hope that this review on the current situation regarding the use of immunoliposomes for the use in cancer chemotherapy provides a realistic perspective on where the field is going and where opportunities can be found for rational improvement of the immunoliposome system. Ideally, two objectives should be met: (1) all administered immunoliposomes should bind to their target epitopes, and (2) all encapsulated drug molecules should become therapeutically available upon binding of immunoliposomes to the target receptors. To date, all targeted systems, including immunoliposomes, fall short of meeting both criteria. However, recent advances have improved the prospects for immunoliposome-based applications. These advances include development of technological solutions for the preparation of long-circulating immunoliposomes, design of 'immunotolerant' targeting ligands, development of new coupling chemistries for attaching targeting ligands to the liposome and attempts to enhance the therapeutic availability of associated drugs. Certainly, encouraging results have been obtained. Over the last 2 decades, major pharmaceutical achievements have been realized in liposomology and this maturing process will facilitate the development of immunoliposome formulations which are pharmaceutically acceptable. In conclusion, immunoliposomes still have a long way to go, but the strong need for more effective chemotherapeutics will continue to motivate studies on immunoliposomes.

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